Effect of ligand binding on the conformation of human plasma vitamin D binding protein (group-specific component)

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Several techniques have been used to demonstrate that the binding of specific ligands to human plasma vitamin D binding protein induces a change in protein conformation. Apoprotein and holoprotein show circular dichroism spectra of similar form in the peptide region with double minima at 207 and 218 nm. The minimum mean residue ellipticity of apoprotein (20.6×10^3 degrees·cm²·dmol⁻¹) is decreased by about 8% after vitamin D₃ binding, suggesting a small change in the backbone conformation. Spectrofluorimetric studies showed that 25-hydroxycholecalciferol causes a saturable enhancement of intrinsic fluorescence of human vitamin D binding protein and alters the pH profile of protein fluorescence, suggesting that there are alterations in the local environment of tryptophan residue(s) after ligand binding. Furthermore, in the presence of 25-hydroxycholecalciferol, the rate of chemical modification of the amino groups in human vitamin D binding protein is decreased and the susceptibility of intact vitamin D binding protein to proteolytic degradation is reduced, suggesting that some surface sites in the vitamin D binding protein molecule are less accessible to external agents. In addition, although the absorbance of vitamin made it difficult to interpret the ultraviolet spectra of holoprotein and apoprotein, the presence of vitamin D binding protein appears to stabilize the vitamin in an aqueous environment, a phenomenon that may be of physiological importance.

In contrast to the chick, which may have separate plasma binding proteins for the vitamins cholecalciferol and 25-hydroxycholecalciferol (D₃ and 25-(OH)-D₃ respectively) (Edelstein *et al.*, 1973), the transport of all vitamin D metabolites in human plasma is mediated by an α -globulin known as group-specific component or Gc† (Daiger *et al.*,

Abbreviations used: D₃, vitamin D₃ or cholecalciferol; Gc, group-specific component; SDS, sodium dodecyl sulphate; vitamin 1,25-(OH)₂-D₃, 1,25-dihydroxycholecalciferol; vitamin 25-(OH)-D₃, 25-hydroxycholecalciferol.

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† Nomenclature for human group-specific component: The present nomenclature follows the recommendations of the Workshop on Group-Specific Component, 17th Congress of the International Society of Haematology, Paris, France, July 1978 (Constans & Cleve, 1979). In this scheme, the three major autosomal alleles of the Gc gene are Gc^{1S}, Gc^{1F} and Gc². The homozygote of each Gc¹ subtype produces two bands on electrophoresis, an anodal band (Gc^{1a}) and a cathodal band (Gc^{1c}), but the Gc² homozygote produces a single electrophoretic band (Gc²).

1975). Human plasma vitamin D binding protein exhibits genetic polymorphism resulting from the existence of three codominant autosomal alleles at a single gene locus (Hirschfeld, 1959; Constans & Cleve, 1979). The protein has been shown to have an isoelectric point of about 4.9 and to consist of a single polypeptide chain of mol.wt. 52 000 (Haddad & Walgate, 1976; Bouillon *et al.*, 1976; Imawari *et al.*, 1976; Svasti *et al.*, 1979a).

The binding of vitamins D₃ or 25-(OH)-D₃ to human vitamin D binding protein decreases the isoelectric point and increases the electrophoretic mobility of the protein (Brissenden & Cox, 1978; Svasti & Bowman, 1978; Van Baelen *et al.*, 1978). Since vitamin D is not charged, such a phenomenon may reflect a vitamin-induced change in the conformation of the binding protein. This idea is supported by the observation that vitamin 25-(OH)-D₃ increases the heat stability of vitamin D binding protein (Bouillon *et al.*, 1976), and by early experiments where vitamin D₃ appeared to enhance the extrinsic fluorescence of a reporter group bound to human vitamin D binding protein (Nilsson *et al.*, 1972), but the latter result is somewhat dubious since the vitamin D binding protein used could not be

immunologically identified as Gc (Petersen, 1971). A change in the conformation of vitamin D binding protein caused by vitamin binding might enable receptor sites on target cells to selectively recognize the holoprotein form of vitamin D binding protein (containing bound vitamin) but not the apoprotein form (Svasti & Bowman, 1978). Such a mechanism would be of great physiological value, because under normal conditions, the concentration of the apoprotein form of vitamin D binding protein is expected to be more than 20-fold that of the holoprotein form. In view of the importance of such a phenomenon, we have used a variety of techniques to definitively show whether conformational changes do occur after ligand binding.

Experimental

Materials

Fresh human plasma and serum were kindly provided by the Thai Red Cross Blood Bank and the Chulalongkorn Hospital Blood Bank (Bangkok, Thailand): they were immediately used or stored at -20°C until use. Crystalline 25-hydroxycholecalciferol monohydrate was a generous gift of Dr. J. Babcock of the Upjohn Chemical Co. (Kalamazoo, MI, U.S.A.). $[1\alpha, 2\alpha\text{-}^3\text{H}]$ Cholecalciferol (sp. activity 12.3 Ci/mmol) and crystalline unlabelled cholecalciferol were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.) and the Sigma Chemical Co. (St. Louis, MO, U.S.A.) respectively. Antisera to human group-specific component and to other human plasma proteins were obtained from Behringwerke A.G. (Hoechst–Thai Co., Bangkok, Thailand). Sephadex products were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and DEAE-cellulose (DE-52) was from Whatman Co. (Maidstone, Kent, U.K.). 2,4,6-Trinitrobenzenesulphonic acid, trypsin and chymotrypsin were purchased from Sigma Chemical Co. Papain was from Worthington Biochemical Co. (Freehold, NJ, U.S.A.). Ammonium sulphate (ultrapure grade) was from Schwarz/Mann Co. (Orangeburg, NY, U.S.A.). Other chemicals were generally of analytical grade.

Purification of human vitamin D binding protein

Human vitamin D binding protein was chromatographically isolated by a modification of our previous procedure (Svasti & Bowman, 1978). Since the starting material in the present experiments was pooled normal serum or pooled normal plasma, rather than the partially purified plasma fractions used previously, it was necessary to include two additional steps in the purification procedure. The two extra steps were $(\text{NH}_4)_2\text{SO}_4$ fractionation (35–55% satn.) added as the first step before

DEAE-Sephadex chromatography and another DEAE-cellulose column (1.6 cm $\times$ 30 cm), eluted with a gradient of 0.05 M–0.15 M-NaCl (120 ml + 120 ml) in 0.05 M-Tris/HCl buffer, pH 7.4, added as the last step after Sephadex G-100 chromatography. Purity was established by electrophoresis in 7.5% polyacrylamide-gel slabs according to the method of Kitchin (1965), with the Tris/borate buffer described by Peacock *et al.* (1965).

Circular dichroism spectroscopy

A 5-fold molar excess of $[1\alpha, 2\alpha\text{-}^3\text{H}]$ cholecalciferol (sp. activity 0.5 mCi/mmol) dissolved in 50–100 μl of ethanol was added to 5–10 mg of human vitamin D binding protein dissolved as a 2.5 mg/ml solution in 0.05 M-NaCl, 0.05 M-Tris/HCl buffer, pH 7.4. After overnight incubation under N_2 , at 4°C in the dark, holoprotein was separated from excess vitamin by chromatography on a DEAE-cellulose column (1.4 cm $\times$ 10 cm), equilibrated with 0.05 M-NaCl, 0.05 M-Tris/HCl buffer, pH 7.4, and successively eluted with 50 ml of the same solvent, followed by 50 ml of 0.2 M-NaCl, 0.05 M-Tris/HCl buffer, pH 7.4. Protein containing fractions were dialysed against distilled water and freeze-dried. Radioactivity was determined in a Packard Tri-carb liquid-scintillation spectrometer with Bray's solution (Bray, 1960). Protein content was determined according to the Lowry procedure (Lowry *et al.*, 1951), with bovine serum albumin as standard, by measuring the absorbance at 600 nm in a Zeiss PMQ II spectrophotometer. The results indicated that the holoprotein preparations contained more than 0.85 mol of vitamin D_3 /mol of protein. Apoprotein controls were treated in the same manner except that $[^3\text{H}]$ vitamin D_3 was omitted during pre-incubation.

Holoprotein and apoprotein were dissolved in 0.1 M-sodium phosphate buffer, pH 6.8, at known protein concentrations (1.0–1.5 mg/ml), determined by the Lowry procedure. C.d. spectra were scanned in a Cary 61 instrument (at the MRC Biophysics Unit, King's College, London, U.K.), with the kind co-operation of Dr. W. B. Gratzer. A mean residue weight of 112 was used to calculate mean residue ellipticity, $[\theta]_{\text{m.r.w.}}$. C.d. spectra were also scanned of vitamin D_3 dissolved at known concentrations in ethanol or in 2% (v/v) ethanol, 0.1 M-sodium phosphate buffer, pH 6.8.

Fluorescence spectroscopy

Fluorescence spectra were scanned in an Aminco-Bowman spectrofluorimeter at an excitation wavelength of 290 nm and an emission wavelength of 340 nm. Fluorescence emission intensity increased linearly up to a protein concentration of 0.1 mg/ml. To observe the effect of vitamin 25-(OH)- D_3 , purified human vitamin D binding protein was dissolved (0.0125 mg/ml) in 0.1 M-NaCl, 5 mM-

sodium phosphate buffer, pH 7.4. Fluorescence spectra were scanned before and after addition of various amounts of vitamin 25-(OH)-D₃ (dissolved in ethanol) to a final molar ratio of vitamin to protein of 1:8 to 2:1. Since vitamin D₃ has a lower affinity for protein than vitamin 25-(OH)-D₃ (Haddad & Walgate, 1976), experiments involving vitamin D₃ were carried out by pre-incubating purified vitamin D binding protein, dissolved (0.25 mg/ml) in the same buffer with varying amounts of vitamin D₃ dissolved in ethanol, overnight under N₂ at 4°C in the dark. Pre-incubated samples were diluted until A_{290} was less than 0.08 before fluorescence measurements. In both cases, the amount of ethanol added with vitamin was always less than 5% (v/v). Controls were also carried out by using an equimolar amount of bovine serum albumin instead of human vitamin D binding protein and by adding ethanol in the absence of vitamin to vitamin D binding protein.

The pH profile of fluorescence was investigated by pre-incubation of purified vitamin D binding protein in distilled water (0.25 mg/ml), with an equimolar amount of vitamin 25-(OH)-D₃ dissolved in ethanol or with the same volume of ethanol, overnight under N₂ at 4°C in the dark. After pre-incubation, the protein solutions were diluted to a final concentration of approx. 0.01 mg/ml with 0.1 M-sodium phosphate buffer, pH varying from 5 to 9. Fluorescence emission at 340 nm was measured at an excitation wavelength of 290 nm.

Ultraviolet absorption spectroscopy

U.v. absorption spectra were scanned in a Beckman Acta V scanning spectrophotometer, with the kind co-operation of Dr. P. Wilairat, Department of Chemistry, Faculty of Science, Mahidol University, Bangkok, Thailand. Human vitamin D binding protein (approx. 2 mg) was dissolved in 2 ml of 0.1 M-NaCl, 5 mM-sodium phosphate buffer, pH 7.4. Protein spectra were scanned before the addition of vitamin and after a 1 h incubation in the dark with an equimolar amount of vitamin 25-(OH)-D₃ (dissolved in 40 µl of ethanol). Spectra were also scanned of the same concentration of vitamin 25-(OH)-D₃ in ethanol and in 2% (v/v) ethanol/0.1 M-NaCl, 5 mM-sodium phosphate buffer, pH 7.4, both freshly prepared and after a 1 h incubation under N₂ in the dark. Difference spectra were scanned by using matched split-cuvettes. The experimental sample contained protein plus vitamin 25-(OH)-D₃ prepared, as described above, in both compartments. In the blank, one compartment contained protein in buffer and the other compartment contained one of the four preparations of vitamin 25-(OH)-D₃ described above (dissolved in ethanol or in 2% (v/v) ethanol/buffer, freshly prepared or incubated for 1 h under N₂ in the dark).

The effect of human vitamin D binding protein on stabilizing vitamin D was also studied. Spectra were scanned at various time intervals of the vitamin 25-(OH)-D₃ added to various solutions compared with the addition of the same volume of ethanol to the same solution as blank. The solutions tested were an equimolar concentration of human vitamin D binding protein in buffer, an equimolar concentration of bovine serum albumin in buffer, buffer alone and ethanol alone. The concentrations of protein, vitamin and buffer were as described. Solutions were kept under N₂ in the dark between measurements.

Chemical modification

Purified vitamin D binding protein, dissolved in water (2 mg/ml), was pre-incubated overnight with a 4-fold molar excess of vitamin 25-(OH)-D₃ dissolved in ethanol or with the same volume of ethanol alone under N₂, in the dark at 4°C. Chemical modification of amino groups with 2,4,6-trinitrobenzenesulphonic acid was carried out according to the procedure of Kute & Westphal (1976). Pre-incubated samples were diluted with 0.05 M-sodium borate buffer, pH 9.0, to a protein concentration of 0.5 mg/ml, and 1.0 ml of 0.1% 2,4,6-trinitrobenzenesulphonic acid was added to 2 ml of each diluted sample contained in matching cuvettes. A_{345} was automatically monitored at 25°C in a Gilford model 2000 recording spectrophotometer with two reagent blanks: 2,4,6-trinitrobenzenesulphonic acid in 0.05 M-sodium borate buffer, pH 9.0, in the presence and absence of vitamin 25-(OH)-D₃. After 8 h, the reaction was completed by heating at 70°C for 15 min.

Proteinase digestion

Purified vitamin D binding protein, dissolved (2 mg/ml) in 0.0625 M-Tris/HCl buffer, pH 6.8, was pre-incubated overnight, with a 4-fold molar excess of vitamin 25-(OH)-D₃ or of cholesterol dissolved in ethanol, under N₂ in the dark at 4°C. Each sample was separately digested with trypsin, chymotrypsin or papain at an enzyme/substrate ratio of 1:50 for 1 h at 37°C; for papain digests, 5 mM-2-mercaptoethanol was included. Digests were stopped by addition of 2% (w/v) SDS and 2-mercaptoethanol to final concentrations at 0.1% and 0.2 M respectively, and heating the mixture to 100°C for 2 min. Peptide fragments were fractionated by electrophoresis in a discontinuous SDS/polyacrylamide gradient [7–15% (w/v) polyacrylamide slab-gel (13 cm × 15 cm × 0.1 cm)] according to the procedure of Laemmli (1970). Electrophoresis was carried out at a voltage of 150 V and a current of 20 mA for 2–3 h; gels were stained in 0.1% Coomassie brilliant blue R.

Results

Isolation of human plasma vitamin D binding protein

Human vitamin D binding protein was isolated from pooled normal plasma or pooled normal serum, which are expected to contain a mixture of all the major Gc proteins (see footnote †). The final product contained Gc², Gc^{1c} and Gc^{1a} in a ratio of approx. 2:4:1 respectively, where the Gc¹ content represents the sum of the protein products of the Gc^{1s} and Gc^{1F} genes, since no attempt was made to distinguish the latter subtypes. This material was free of contamination by other proteins as judged by polyacrylamide-gel electrophoresis and by immunoelectrophoresis with rabbit antiserum to human whole serum. The relatively low ratio of Gc^{1a} to Gc^{1c} obtained reflects the stringent pooling of tubes in DEAE-cellulose chromatography, at pH 8.8, that is necessary to obtain a product free of α_1 -antitrypsin contamination. The higher content of total Gc¹ proteins to total Gc² reflects the higher gene frequency of Gc¹ in the Thai population (Rucknagel *et al.*, 1968). The use of a mixture of Gc proteins is unlikely to pose any serious problems since the Gc proteins probably do not differ greatly in affinity for vitamin (Bouillon & Van Baelen, 1979) and show only minor differences in amino acid sequence and carbohydrate content (Svasti *et al.*, 1979b). Furthermore, we have not attempted to remove vitamin D from our preparations of vitamin D binding protein before carrying out the present experiments because the % saturation of vitamin D binding protein in normal plasma is $\leq 5\%$. In any case, the presence of small amounts of vitamin in our apoprotein preparation would tend to minimize the differences detectable between holoprotein and apoprotein rather than give any artificial difference not normally present.

Circular dichroism spectroscopy

In the peptide bond region (200–250nm), both apoprotein and holoprotein show a double minimum at 207 and 218nm of substantial magnitude (Fig. 1a), indicating the presence of significant amounts of α -helix. The magnitude of the negative ellipticity of vitamin D binding protein between 207 and 223nm is slightly decreased after vitamin D₃ binding, similar to the effect observed upon the binding of progesterone binding globulin (Stroupe & Westphal, 1975). The c.d. spectra of human vitamin D binding protein in the aromatic region (255–300nm) are not shown because the low magnitude of negative ellipticity made the spectra somewhat noisy and because it was difficult to select the appropriate blank to compensate for the significant contribution of vitamin D₃ to the holoprotein spectrum (for

Fig. 1. C.d. spectra of human vitamin D binding protein in peptide region

Holoprotein (more than 85% of vitamin D₃ bound) and apoprotein were dissolved at known protein concentrations in 0.1M-sodium phosphate buffer, pH6.8. C.d. spectra were scanned in a Cary 61 instrument. Mean residue ellipticity, $[\theta]_{m.r.w.}$, for holoprotein and apoprotein were calculated assuming a mean residue weight of 112. The spectrum of vitamin D₃ (at the same concentration as protein) in ethanol was also determined and was normalized with the same correction factor used in the calculations of protein ellipticity. —, Holoprotein; ----, apoprotein; ····, ellipticity contribution of vitamin D₃.

further discussion, see under 'Ultraviolet absorption spectroscopy' in the Results section).

Fluorescence spectroscopy

Although the amino acid sequence of human vitamin D binding protein has not yet been established, amino acid analyses indicate that the protein contains approx. 16–18 tyrosine and 2–5 tryptophan residues per mol (Bouillon *et al.*, 1976; Imawari *et al.*, 1976). The fluorescence spectrum of apoprotein (Fig. 2) is typical of proteins containing tryptophan, showing excitation and emission maxima at 290 and 340nm respectively. In the presence of vitamin D₃, there is an enhancement in the intrinsic fluorescence of human vitamin D

Fig. 2. Fluorescence excitation and emission spectra of human vitamin D binding protein

Fluorescence spectra were scanned in the presence and absence of an 8-fold molar excess of vitamin D₃ in an Aminco-Bowman spectrofluorimeter. Protein was dissolved (0.0125 mg/ml) in 5 mM-sodium phosphate buffer/0.1 M-NaCl, pH 7.4. Excitation spectra were scanned at an emission wavelength of 340 nm and emission spectra were scanned at an excitation wavelength of 290 nm. —, Vitamin D binding protein + vitamin D₃; ---, vitamin D binding protein alone; ..., vitamin D₃ in ethanol.

binding protein of about 20% without any detectable shift in the wavelength maxima for emission or excitation (Fig. 2). Vitamins D₃ or 25-(OH)-D₃ alone do not exhibit fluorescence at these wavelengths. Addition of ethanol alone to purified human vitamin D binding protein does not affect the spectrum of the apoprotein. Furthermore, addition of vitamins D₃ or 25-(OH)-D₃ to human immunoglobulins or to bovine serum albumin does not affect the fluorescence spectra of these proteins, even though it has been reported that serum albumin can bind vitamin D derivatives in a non-specific manner (Silver & Fainaru, 1979).

Thus the observed fluorescence enhancement appears to result from the specific interaction of vitamin D with its binding protein. This is further supported by titration experiments involving vitamin 25-(OH)-D₃, where fluorescence enhancement reaches a saturating maximum at a molar ratio of vitamin to protein of 1 : 1 (Fig. 3), in agreement with the reported 1 : 1 stoichiometry of binding (Haddad & Walgate, 1976; Bouillon *et al.*, 1976). Higher molar ratios of vitamin to protein are required for saturation of fluorescence with vitamin D₃, consistent with the lower affinity of vitamin D binding protein for vitamin D₃ compared with vitamin 25-(OH)-D₃ (Haddad & Walgate, 1976). We are presently unable to explain the slight decrease in fluorescence observed when more than equimolar concentrations of vitamin 25-(OH)-D₃ are used since

Fig. 3. Fluorescence titration of human vitamin D binding protein

Increasing amounts of vitamin 25-(OH)-D₃, dissolved in ethanol, were added to a 0.0125 mg/ml solution of human vitamin D binding protein in 5 mM-sodium phosphate buffer/0.1 M-NaCl, pH 7.4. Fluorescence emission at 340 nm was measured in an Aminco-Bowman spectrofluorimeter at an excitation wavelength of 290 nm.

a similar effect was not observed with serum albumin.

The fluorescence enhancement observed here contrasts with the fluorescence quenching observed on the binding of progesterone to progesterone binding globulin (Stroupe *et al.*, 1975). The same degree of enhancement is observed when excitation wavelengths of 295 and 305 nm are used. Since tyrosine absorption is negligible at these wavelengths (Teale, 1960), the enhancement observed is likely to involve tryptophan residues. Enhancement of tryptophan fluorescence may typically be due to a change in the local environment of the fluorescent group(s), from a more polar to a less polar environment, or a dequenching effect resulting from the relative movement of a tryptophan residue and a quencher group away from each other. Fluorescence enhancements are normally accompanied by a shift in the wavelength maxima to the blue end of the spectrum. Although no shifts in wavelength were observed in the present experiments, small shifts in wavelength are more difficult to detect than small changes in fluorescent intensity. In addition, since human vitamin D binding protein contains more than one tryptophan, it is possible that the observed enhancement is the combined effect of small changes in the local environment in several tryptophan

residues, making the shift in wavelength maxima too small to detect.

The pH profile of vitamin D binding protein fluorescence was also studied in the presence and absence of the vitamin 25-(OH)-D₃. The results (Fig. 4) showed that, in the absence of vitamin, the intrinsic fluorescence of vitamin D binding protein changed very little in the pH range between pH 5.5 and 8.5. On the other hand, the fluorescence of vitamin D binding protein in the presence of vitamin falls dramatically when the pH is lowered from pH 7.5 to 6.0. These results are similar to those observed upon the binding of the competitive inhibitor tri-*N*-acetylglucosamine to the enzyme lysozyme (Lehrer & Fasman, 1966), which has been explained by an increase in proximity between Glu35 (of abnormally high pK_a) and Trp 108, as a result of inhibitor binding (Lehrer, 1976). In the absence of information on the tertiary structure of human vitamin D binding protein, it would not be profitable to speculate further on the present results except to say that the binding of vitamin appears to be associated with a rearrangement of quencher groups in the protein. In the absence of vitamin, the

quenching of vitamin D binding protein fluorescence is not associated with any group that ionizes in the pH range 5.5 to 8.5. But in the presence of the vitamin 25-(OH)-D₃, the quenching of vitamin D binding protein fluorescence appears to be associated with the protonation of a group with a pK_a of 6.5, for example histidine.

Ultraviolet absorption spectroscopy

The u.v. absorption spectrum of human vitamin D binding protein in the aromatic region shows maximum absorption at 274 nm (Fig. 5). After a 1 h incubation with an equimolar amount of vitamin 25-(OH)-D₃, the absorbance of vitamin D binding protein is increased by approx. 25% without any major change in the wavelength maximum (Fig. 5). Unfortunately, vitamin 25-(OH)-D₃ itself shows significant absorbance and the spectrum obtained depends considerably on the solvent (Fig. 5); however, light scattering was negligible at the low concentrations of vitamin used. In a 2% (v/v) ethanol/buffer solution, the absorbance of vitamin decreases with time (Fig. 5), suggesting that the vitamin is unstable under these conditions. But in ethanol there is no appreciable change in the vitamin spectrum with time (not shown). The reasons for this instability are unclear because all solutions were maintained under N₂ in the dark, but it is possible that solutions in 2% (v/v) ethanol/buffer contained more dissolved oxygen than solutions in ethanol.

Difference spectra were also scanned by using split cuvettes, with the blank containing, in one compartment, vitamin D binding protein dissolved in buffer and, in the other compartment, one of three equimolar 25-(OH)-D₃ solutions: dissolved in ethanol, freshly prepared in 2% (v/v) ethanol/buffer, or incubated for 1 h under N₂ in the dark in 2% (v/v) ethanol/buffer. The difference spectra obtained with the three blanks were all different and are not shown here, because it was not possible to decide which blank was the most appropriate. Arguments could be for the solution in ethanol on the grounds that the vitamin may not be completely soluble in 2% (v/v) ethanol/buffer, or for the use of 2% (v/v) ethanol/buffer since this is the solvent in the experimental cuvette. In the latter case, a freshly prepared solution would be advocated if binding was 100% and if protein-bound vitamin was completely stable, whereas a pre-incubated solution would be recommended if protein-bound and free vitamin were equally unstable.

Because of the problems in interpreting the effect of vitamin binding on the u.v. spectrum of vitamin D binding protein, we have instead focused our attention on the effect of vitamin D binding protein on the spectrum of vitamin. As noted above, vitamin 25-(OH)-D₃ does not appear to be stable in 2% (v/v) ethanol/buffer. The presence of an equimolar con-

Fig. 4. pH profile of human vitamin D binding protein fluorescence

Purified vitamin D binding protein (0.25 mg/ml) dissolved in water was pre-incubated with an equimolar concentration of the vitamin 25-(OH)-D₃ (dissolved in ethanol) or with ethanol alone, under N₂ in the dark at 4°C overnight. Both samples were diluted to approx. 0.01 mg/ml with 0.1 M-sodium phosphate buffer, pH varying from 5 to 9. Fluorescence emission at 340 nm was measured in an Aminco-Bowman spectrofluorimeter at an excitation wavelength of 290 nm. ●—●, Vitamin D binding protein + vitamin 25-(OH)-D₃; ○----○, vitamin D binding protein alone.

Fig. 5. U.v. absorption spectra of human vitamin D binding protein and vitamin 25-(OH)-D₃

U.v. absorption spectra were scanned in a Beckman Acta V spectrophotometer. Human vitamin D binding protein was dissolved (2 mg/2 ml) in 0.1 M-NaCl, 5 mM-sodium phosphate buffer, pH 7.4. Protein spectra were scanned before the addition of vitamin and after a 1 h incubation in the dark with an equimolar amount of vitamin 25-(OH)-D₃ added in 0.04 ml of ethanol. Spectra were also scanned of an equimolar amount of vitamin 25-(OH)-D₃ in ethanol or in 2% (v/v) ethanol/0.1 M-NaCl, 5 mM-sodium phosphate buffer, pH 7.4 (freshly prepared or incubated for 1 h in the dark). (a) Vitamin D binding protein + vitamin 25-(OH)-D₃; (b) vitamin D binding protein alone; (c) vitamin 25-(OH)-D₃ in ethanol; (d) fresh vitamin 25-(OH)-D₃ in 2% (v/v) ethanol/buffer; (e) vitamin 25-(OH)-D₃ incubated in 2% (v/v) ethanol/buffer for 1 h in the dark under N₂.

centration of bovine serum albumin does not appear to improve the stability of vitamin 25-(OH)-D₃ (Fig. 6a). However, the presence of an equimolar concentration of human vitamin D binding protein markedly increases the stability of 25-(OH)-D₃ in aqueous solution, as judged by the much slower decrease in absorbance observed (Fig. 6b). This stabilization of vitamin may also be of physiological significance.

Fig. 6. U.v. spectra of vitamin 25-(OH)-D₃ in protein solutions at various times

Human vitamin D binding protein was dissolved (2 mg/2 ml) in 0.1 M-NaCl, 5 mM-sodium phosphate buffer, pH 7.4. An equimolar concentration of bovine serum albumin was also prepared in the same buffer. An equimolar amount of 25-(OH)-D₃ in 0.04 ml of ethanol was added to the vitamin D binding protein and albumin solutions. Spectra of vitamin were scanned in a Beckman Acta V spectrophotometer at various times with the appropriate protein solution, after addition of the same volume of ethanol as blanks. Vitamin solutions were maintained at 25°C in the dark under N₂ between measurements. (a) Vitamin 25-(OH)-D₃ spectrum in the presence of bovine serum albumin: —, at 2 min; ---, at 30 min; ···, at 2 h. (b) Vitamin 25-(OH)-D₃ spectrum in the presence of human vitamin D binding protein: —, at 2 min; ---, at 30 min; ···, at 3 h.

Chemical modification

The presence of vitamin 25-(OH)-D₃ decreases the rate of chemical modification of amino groups in vitamin D binding protein by 2,4,6-trinitrobenzenesulphonic acid at 25°C (Fig. 7). After 6 h, the number of amino groups modified per mol of protein was 21.3 in the presence of vitamin and 25.0 in the absence of vitamin. But after heating at 70°C for 15 min to complete the reaction, the total number of amino groups modified in the presence and absence of vitamin was similar: this may be due to denaturation of protein at the higher temperature, resulting in the loss of vitamin. This vitamin induced protection of amino groups from chemical modification is similar to the effect observed upon the binding of progesterone to progesterone binding globulin (Westphal, 1977). This phenomenon does

Fig. 7. Chemical modification of amino groups with 2,4,6-trinitrobenzene sulphonic acid

Purified vitamin D binding protein (2 mg/ml) dissolved in water, was incubated with a 4-fold molar excess of the vitamin 25-(OH)-D₃ or with ethanol under N₂ in the dark, at 4°C overnight. After pre-incubation, 2 ml of each sample, after dilution (to 0.5 mg/ml) in 0.5 M-sodium borate buffer, pH 9.0, was reacted with 1 ml of 0.1% 2,4,6-trinitrobenzene sulphonic acid was monitored in a Gilford model 2000 recording spectrophotometer, with two reagent blanks, one including and one not including 25-(OH)-D₃. After 8 h, the reaction was completed by heating at 70°C for 15 min. ●—●, Vitamin D binding protein + vitamin 25-(OH)-D₃; ○----○, vitamin D binding protein alone.

not necessarily imply that amino groups are involved in vitamin binding and may result from a decreased surface accessibility caused by the binding of vitamin.

Proteinase digestion

Although digestion of native human vitamin D binding protein with pepsin or with pronase was very extensive, digestion with chymotrypsin, papain or trypsin yielded larger fragments detectable by SDS/polyacrylamide-gel electrophoresis (Fig. 8). Very little digestion was observed with chymotrypsin and no difference could be detected between samples incubated with cholesterol or with vitamin 25-(OH)-D₃ (Fig. 8; slots 3, 4). With trypsin (Fig. 8; slots 1, 2) and with papain, vitamin D binding protein pre-incubated with cholesterol gave a different peptide pattern to vitamin D binding protein pre-incubated with vitamin 25-(OH)-D₃. With both enzymes, vitamin appears to have a protective effect on digestion, since samples pre-incubated with vitamin 25-(OH)-D₃ contained larger amounts of high-molecular-weight material and smaller amounts of low-molecular-weight material than samples pre-incubated with cholesterol. However, the presence of vitamin appeared to decrease

Fig. 8. Proteinase digestion products of human vitamin D binding protein analysed by SDS/polyacrylamide-gel electrophoresis

Purified vitamin D binding protein was dissolved as a 2 mg/ml solution in 0.0625 M Tris/HCl buffer, pH 6.8. One sample (+) was incubated with a 4-fold molar excess of 25-(OH)-D₃, under N₂ in the dark at 4°C overnight, while another sample (–) was similarly incubated with a 4-fold molar excess of cholesterol. After preincubation, each sample was digested with trypsin (T), chymotrypsin (C) or papain (P). Digests were analysed by SDS/polyacrylamide-gel electrophoresis at a voltage of 150 V and a current of 20 mA for about 2–3 h. The gel was stained with Coomassie brilliant blue R. (1) Vitamin D binding protein, digested with trypsin; (2) vitamin D binding protein + vitamin 25-(OH)-D₃, digested with trypsin; (3) vitamin D binding protein, digested with chymotrypsin; (4) vitamin D binding protein + vitamin 25-(OH)-D₃, digested with chymotrypsin; (5) vitamin D binding protein, digested with papain; (6) vitamin D binding protein + vitamin 25-(OH)-D₃, digested with papain.

the rate of digestion rather than to generate new digestion products. The protective effect of vitamin on the proteolytic degradation of human vitamin D binding protein may also be due to a vitamin-induced conformational change that limits the accessibility of some of the susceptible bonds.

Discussion

The present studies conclusively show that the binding of vitamin causes a change in the conformation of human vitamin D binding protein, which is manifested as a small but reproducible change in the backbone conformation detectable by c.d. spectroscopy, as a change in the environment of aromatic residues (particularly tryptophan), detectable by fluorescence spectroscopy, and as re-

arrangements of surface residues, detectable by chemical modification and proteinase digestion experiments. As noted earlier, such changes in conformation may provide a mechanism for preventing surface receptors on target cells from becoming saturated with excess apoprotein. In this connection, a change in the accessibility of surface residues is particularly interesting since interaction of vitamin D binding protein with cell receptors is likely to involve residues at the surface of the molecule.

Although vitamin D binding proteins have also been found in tissues, the mechanism of action of plasma vitamin D binding protein remains uncertain. It is now generally agreed that the 5–6S tissue vitamin D-binding protein found in all nucleated tissues results from the interaction of plasma vitamin D binding protein with a 4S cytosolic protein lacking the capacity to bind vitamin (Van Baelen *et al.*, 1977; Kream *et al.*, 1979; Cooke *et al.*, 1979). This protein has been shown to be identical to actin, a constituent of all nucleated cells (Van Baelen *et al.*, 1980), making it less likely that the protein is involved in the transport or intracellular action of antirickettial sterols. Another soluble tissue-binding protein (3.5–3.7S) has been demonstrated to occur in several target tissues (Brumbaugh & Haussler, 1974; Haddad *et al.*, 1976; Kream *et al.*, 1977; Weckslar *et al.*, 1979). Since this protein appears to bind vitamin 1,25-(OH)₂-D₃ preferentially compared with vitamin 25-(OH)-D₃, it may be the binding protein responsible for mediating the intracellular action of vitamin D. However, there have been no reports of binding between this protein and plasma vitamin D binding protein. It is, therefore, entirely possible that plasma vitamin D binding protein may interact with another receptor at the cell surface before vitamin can be transferred to this intracellular 3.5–3.7S binding protein. In such an event, the conformational changes reported here suggest that a promising experimental approach to isolate this putative cell-surface receptor might be to look for membrane proteins in the intact cell that are able to bind the holoprotein form of plasma vitamin D binding protein and not the apoprotein form.

The high concentrations of vitamin D binding protein in human plasma compared to the total concentration of vitamin D metabolites has raised the question of whether the biological function of vitamin D binding protein is only transport *per se*. Thus suggestions have been made that vitamin D binding protein may assist the conversion of pre-vitamin to vitamin in the skin (Holick *et al.*, 1979), play a role in preventing both vitamin D deficiency and vitamin D toxicity (Bouillon & Van Baelen, 1979) or assist in depolymerizing actin released into plasma (Van Baelen *et al.*, 1980). The present u.v. spectroscopic studies indicate that stabilization of

vitamin in an aqueous environment is another function that should be considered.

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